Bacterial rhizoplane colonization patterns of *Buchloe dactyloides* growing in metalliferous mine tailings reflect plant status and biogeochemical conditions

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Abstract

Plant establishment during phytostabilization of legacy mine tailings in semiarid regions is challenging due to low pH, low organic carbon, low nutrients, and high toxic metal(loid) concentrations. Plant-associated bacterial communities are particularly important under these harsh conditions because of their beneficial services to plants. We hypothesize that bacterial colonization profiles on rhizoplane surfaces reflect deterministic processes that are governed by plant health and the root environment. The aim of this study was to identify associations between bacterial colonization patterns on buffalo grass (*Buchloe dactyloides*) rhizoplanes and both plant status (leaf chlorophyll and plant cover) and substrate biogeochemistry (pH, electrical conductivity, total organic carbon, total nitrogen, and rhizosphere microbial community). Buffalo grass plants from mesocosm- and field-scale phytostabilization trials conducted with tailings from the Iron King Mine and Humboldt Smelter Superfund Site in Dewey-Humboldt, Arizona were analyzed. These tailings are extremely acidic and have arsenic and lead concentrations of 2 4 g kg^{-1} substrate. Bacterial communities on rhizoplanes and in rhizosphere-associated substrate were characterized using fluorescence *in situ* hybridization and 16S rRNA gene amplicon sequencing, respectively. The results indicated that the metabolic status of rhizoplane bacterial colonizers is significantly related to plant health. Principal component analysis revealed that root-surface *Alphaproteobacteria* relative abundance was associated most strongly with substrate pH and *Gammaproteobacteria* relative abundance associated strongly with substrate pH and plant cover. These factors also affected the phylogenetic profiles of the associated rhizosphere communities. In summary, rhizoplane bacterial colonization patterns are plant specific and influenced by plant status and rhizosphere biogeochemical conditions.

Keywords

Phytostabilization; rhizosphere; rhizoplane; plant-microbe associations; FISH; 16S rRNA gene amplicon sequencing

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Introduction

Metal(loid) contamination due to anthropogenic activities, such as mining, poses a threat to the surrounding environment and human health. In particular, legacy mine tailings, which consist of the residual ore left behind after mining at historic sites, are susceptible to wind and water erosion, facilitating contaminant transport. Revegetation can be exploited to reduce the spread of contaminants via wind and water erosion, but technologies must be developed to facilitate plant growth in these harsh materials. Typically a barrier such as a soil cap has been used to isolate mine tailings from plants [1], however, phytostabilization provides an alternative method in which a plant cover is established directly into the tailings without a soil cap to promote the immobilization of metal(loid)s in the root zone [2]. In addition to being less costly [2,3], phytostabilization is less environmentally destructive because it does not necessitate soil excavation from surrounding lands. However, plant establishment during phytostabilization can be difficult, particularly in weathered pyritic mine tailings under semiarid conditions, due to acidic pH, low organic carbon content, low nutrients, hypersalinity, low moisture, poor substrate structure, and an autotroph-dominated, acid-generating, microbial community [2,4,5,6,7]. Organic amendments are typically required, such as compost, manure, or biosolids, to provide a source of organic carbon and nutrients, pH neutralization, increased water holding capacity, improved aggregate structure, and the addition of a heterotrophic microbial inoculum [2,4,8,9].

Addition of the heterotrophic microbial inoculum is important because a strong correlation has been observed between the abundance of neutrophilic heterotrophic bacteria and plant biomass for plants grown in acidic, pyritic mine tailings [10]. Research focused on specific plant-root-microbe interactions during phytostabilization of pyritic mine tailings is essential to advance our understanding of how plant-microbe interactions help sustain plants under such stressful environmental conditions. Two areas in which there is direct interaction between plants, microorganisms, and substrates (mine tailings material) are the rhizoplane and rhizosphere which are defined as the root surface and root-exudate impacted soil surrounding the root, respectively. Factors governing rhizoplane bacterial colonization, particularly of plants growing in mine tailings, are poorly understood. Fluorescence in situ hybridization (FISH) in combination with confocal scanning electron microscopy (CLSM) has been used to study bacterial rhizoplane-colonization in typical soils [11,12,13] and has been adapted for roots grown in compost-amended mine tailings [14,15,16,17]. In a comparison of *Buchloe dactyloides* (buffalo grass) grown in unamended vs compost-amended (10%) mine tailings, increases were observed for both percent bacterial coverage of the rhizoplane and plant biomass (3.6 to 18.9% and 0.045 g pot^{-1} to 0.18 g pot^{-1}, respectively) with compost amendment [16]. These results show an association between domain-level bacterial root colonization and either external substrate or plant condition.

The microbial ecology of the rhizosphere has been studied more extensively. Root exudates consisting of organic compounds such as polysaccharides, sugars, amino acids, and fatty acids [18,19,20,21] create the “rhizosphere effect” that is characterized by a 10- to 100-fold greater abundance of microbes in the rhizosphere as compared to the bulk soil [22]. Previous research does indicate that rhizosphere bacteria, specifically plant growth promoting bacteria (PGPB), are particularly important for plant growth and establishment under extreme

conditions of the mine tailings environment [14,15,23,24,25,26,27]. PGPB enhance plant growth by 1) increasing nutrient availability through phosphorous solubilization, production of iron-chelating siderophores, and nitrogen fixation; 2) decreasing the plant-stress hormone ethylene through production of ethylene-cleaving ACC (1-aminocyclopropane-1-carboxylate) deaminase; 3) providing protection from pathogens; and 4) decreasing metal bioavailability to plants [28,29,30]. These services are crucial in the nutrient-limited, stressed conditions of mine tailings and facilitate positive plant-microbe feedbacks.

In this study we evaluate rhizoplane and rhizosphere bacterial communities of buffalo plants grown in pyritic tailings from the Iron King Mine and Humboldt Smelter Superfund (IKMHSS) site in Dewey-Humboldt, AZ from previously described mesocosm [7] and field trial [31] experiments. We contend that understanding the factors controlling rhizoplane microbial colonization patterns will provide important insights into the significance of plant-microbe associations to plant survival during phytostabilization of acidic mine tailings. FISH and CLSM were used to analyze buffalo grass rhizoplane bacterial colonization, and associations were found between colonization patterns and both substrate biogeochemistry and plant status. We hypothesized that processes driving rhizoplane bacterial colonization are not stochastic, but are determined, at least in part, by both plant status and external biogeochemical parameters. Therefore, the aims of this study were to: i) quantify bacterial rhizoplane colonization of buffalo grass grown in compost-amended IKMHSS mine tailings at the domain and phylum/subphylum-level; ii) identify associations between bacterial rhizoplane colonization and both plant status (leaf chlorophyll and plant cover) and geochemical parameters (pH, total organic carbon [TOC], total nitrogen [TN], and electrical conductivity [EC]); and iii) evaluate associations between rhizoplane colonization patterns and rhizosphere microbial community composition, profiled by high throughput sequencing of 16S rRNA gene amplicons. This study provides new insights into associations between bacteria colonizing the rhizoplane and plant status in the stressful mine tailing environment.

Methods

Iron King Mine and Humboldt Smelter Superfund Site Description

The Iron King Mine and Humboldt Smelter Superfund (IKMHSS) site is located in the town of Dewey-Humboldt, AZ. Mining operations were carried out between 1906 and 1969 extracting Cu, Ag, and Au [32], but leaving high residual tailings concentrations of toxic metal(loid)s including As and Pb, at 3.1 and 2.2 g kg^{-1} tailings respectively [33]. Oxidation of the pyritic tailings in the top 25 cm has produced acidic conditions (pH of 2.3 2.7) [6]. The tailings are characterized by low organic carbon content (0.14 g kg^{-1}), hypersalinity (EC 6.5–9.0 ds m^{-1}), poor substrate structure [6,7], and the complete absence of vegetation. Both a controlled 1-year greenhouse mesocosm study [7,34] and a 7-year field trial [31] have been conducted to develop phytostabilization strategies for heavy metal containment at this site. This study examines plant-microbe relationships for buffalo grass plants grown in IKMHHS tailings from both the mesocosm study and field trial, as described below.

Mesocosm Study

Controlled Greenhouse Experiment—Buffalo grass plants were harvested to assess bacterial rhizoplane colonization patterns from a previously established controlled, greenhouse mesocosm experiment [7,34]. The mesocosm experiment was designed to evaluate phytostabilization of IKHMSS mine tailings in a controlled system undergoing defined acidification. The 12-month greenhouse experiment was conducted at the Controlled Environment Agriculture Center at the University of Arizona in highly instrumented polypropylene mesocosms that were 1 m in diameter and 0.5 m deep and instrumented with pore water samplers at 10 cm intervals from 5 35 cm along the depth of the mesocosm. Buffalo grass plants analyzed for this study were grown in tailings amended with 15% compost (w/w) and harvested over the first 9 months of the mesocosm experiment. Compost was incorporated into the top 20 cm of tailings of the mesocosms prior to seeding and plants were irrigated at a rate of 5–10 L per week. Germination was observed within the first week of the experiment and plants grew well for 3 months. After 3 months, plants showed signs of stress and severe die-back was observed by 12 months.

Root Sample Collection—Buffalo grass plants were harvested from three replicate mesocosms at 3, 6, and 9 months following planting and the roots collected as follows. The top of the plant was cut off at the surface of the substrate and then a corer measuring 2 cm in diameter and 10 cm in length was inserted directly over the truncated plant to enable sampling of the root plus rhizosphere substrate [7]. A small section of root sample was cut off and treated to fix bacteria colonizing the rhizoplane, as described previously [16,17]. Briefly, the root sections were placed into 4 °C, sterile 4 % paraformaldehyde (PFA) in phosphate-buffered saline solution (PBS; 7 mM Na_2HPO_4, 3 mM $NaHPO_4$ and 130 mM NaCl, pH 7.2) and processed in the laboratory within 12 h of collection as follows. Roots were rinsed twice in phosphate buffered saline (PBS), then stored in a 1:1 mixture of PBS and 100 % ethanol at −20 °C until FISH analysis, which was performed within 2 years of sample collection.

Mesocosm Plant Status (Plant Cover) and Pore Water Geochemical Parameters—Plant cover was determined for each of the buffalo grass mesocosms at each of the collection times (3, 6, and 9 months). A photograph of each mesocosm at the specified time was imported into the program ImageJ [35] for digital analysis of percent plant cover.

Pore water was collected continuously at 5 and 15 cm depths and the depths were consolidated for chemical analysis. Pore water subsamples collected at 0, 3, 6, and 9 months were analyzed for pH, dissolved organic carbon (DOC), dissolved nitrogen (DN), and electrical conductively (EC) as described previously [7,34].

Field Study

IKHMSS Phase 1 and Phase 3—A total of 15 buffalo plants were also harvested for analysis of root colonization patterns from a long-term IKHMSS field trial. The field trial consists of 3 individual experiments, or phases, that were designed to evaluate multiple direct planting strategies for native plant species in compost-amended tailings, as described in detail in Gil-Loaiza et al., [31]. Phase 1, initiated in May 2010, was designed to study

different compost amendment rates and was seeded with a mixture of six native plant species in semi-random plots. For the current bacterial rhizoplane colonization study, 8 buffalo grass roots from the 15% compost (w/w in the top 20 cm) treatment were examined. Phase 3 of the field trial was initiated in June 2012 and was designed to study plant establishment of buffalo grass or quailbush monocultures situated in alternating rows. The entire area was amended with 15% compost (w/w in the top 20 cm) and lime (2.0622 kg m^{-2}). For the current bacterial rhizoplane colonization study, 7 buffalo grass plants from the 3 replicates of the buffalo grass treatment were examined. With the exception of the treatment differences listed above, Phase 1 and Phase 3 field experiments were established according to the same procedures. Specifically, the Phase 3 differences included amendment with lime, seeding of buffalo grass as a monoculture rather than a seed-mix of 6 plant species, and a row configuration rather than a semi-random plot design.

Sample Collection—Eight buffalo grass plants were sampled from Phase 1 that included two plants from each of the four replicate plots for the 15%-compost treatment (labelled as follows: Plot 5a and b, Plot 10a and b, Plot 19a and b, and Plot 24a and b). Eight plant were also sampled from the three replicate rows of the 15%-compost, buffalo grass treatment of Phase 3 (labelled as follows: Row 2a and b; Row 4a, b, and c; and Row 6a, b, and c). All plants were harvested in August 2013. After collection, root samples were fixed immediately, as described above. In addition, following the removal of the root sample, the entire core was placed in a sterile bag for rhizosphere DNA analysis. Corers were sterilized with 95% ethanol before each sample collection. Additionally, a surface tailings sample from the top 20 cm was collected next to the root and stored in a plastic bag for substrate geochemical analysis.

Field Study Plant Status and Substrate Geochemical Analysis—The following field measurements were used to quantify plant establishment and status. First, as a measure of plant establishment, plant canopy cover was estimated using transect and quadrant sampling. Plant canopy cover measurements were done in October 2013, 2 months after buffalo grass root and rhizosphere collections, for each Phase 1 field plot and Phase 3 row section from which a plant was harvested [36,37] by Gil-Loaiza et al. [31]. Second, leaf chlorophyll levels were measured for each plant as an indication of individual plant stress [38,39,40] at the time of root and rhizosphere collection using a handheld portable chlorophyll meter (atLEAF; Wilmington, DE) which measures light transmittance. Values produced by the meter are unit-less indices [41, 42], in which higher measured values correspond to healthier plants within the same species [40].

Surface tailings collected next to each plant were analyzed for pH, TOC, TN, and EC. Each surface sample was sieved at 2 mm and dried at 65 ° C for 72 h. The pH and EC were measured from a 1:2 mass ratio extract of tailings to ultrapure DI water (18.2 MΩ, Milli-Q) that was mixed for 30 minutes prior to measurement. The pH was measured in the homogenized extract and the EC was determined from the supernatant after the substrate was allowed to settle for 10–15 minutes. Analyses of TOC and TN were performed on milled subsamples of the dried rhizosphere material (Shmiadzu TOC analyzer, Columbia, MD) using a solid state module (SSM), which utilizes a dry combustion under oxygen with

detection by non-dispersive infrared (NDIR) for total carbon and chemoluminescence for TN.

Fluorescence in situ Hybridization (FISH)

Bacterial rhizoplane colonization of buffalo grass roots was assessed using FISH with probes chosen to target the dominant phyla/subphyla present in the IKHMSS buffalo grass rhizosphere, as described in detail in Honeker et al., [17]. *Proteobacteria* and *Actinobacteria* were the dominant phyla present at 42.7% and 15% relative abundance, respectively. Within the *Protoebacteria* phylum*, Alphaproteobacteria* and *Gammaproteobacteria* were most abundant at relative abundances of 22.4% and 14.2 %, respectively [17]. The FISH protocol was adapted from Watt et al. [11] and Iverson and Maier [16] and is described in detail in Honeker et al. [11]. Briefly, a fixed root was immobilized on a glass slide and incubated in hybridization buffer with fluorescently-labelled probes (Eub338-CY5 mix to target the domain Bacteria plus one of the following specific probes: HGC69a-CY3 for *Actinobacteria*, Alf968-CY3 for *Alphaproteobacteria,* or Gam42a-CY3 for *Gammaproteobacteria*; probe sequences are from ProbeBase [43]) for 2–2.5 h at 46 °C followed by incubation in wash buffer for 15 min at 48 °C. Slides with roots were rinsed in ice-cold DI water, dried using compressed air, and then placed in the dark at room temperature until completely dry. Slides were stored in a dark slide box with desiccant at −20 °C until viewing.

Confocal Laser Scanning Microscopy (CLSM)

Prior to viewing, slides were allowed to equilibrate to room temperature. As a counterstain, 40 μL of 500 μM SytoBC nucleic acid stain (Molecular Probes, OR) in 10 μM Tris-HCl at pH 8.0 was added to each root and incubated at room temperature for 20 min. SytoBC was rinsed off with 10 μM Tris-HCl and excess liquid was removed by wicking with a Kimwipe and gently blowing with condensed air. The slides were then placed in the dark at room temperature until completely dry.

Once dry, 1 drop of AF1 antifadent mountant (Citifluor Ltd., London) was added to the root and then a coverslip (size 1.5, VWR) was placed over the root. Slides were viewed on a Zeiss 510 Meta Confocal Laser Scanning Microscope (CLSM) using a 63x objective (Plan Apochromat, NA 1.3). For viewing SytoBC, CY3 labeled probes, and CY5 labeled probes, a 488nm Argon laser with a BP 505–570 filter, 543nm HeNe laser with an LP 560 filter, and 633 nm HeNe laser with an LP 650 filter were used, respectively. The captured image consisted of three channels, each representing a different wavelength, and hence, a different probe or the nucleic acid stain (see Fig. 1 for a representative set of images). Each probe has a specific excitation and emission wavelength, and the three laser wavelengths and filters listed were optimally chosen to ensure excitation and image collection of the appropriate probe while avoiding any overlap of probe excitation and emission wavelengths. Images were collected at 1 μm intervals from the surface of the root to a depth of 6 μm.

Quantitative Image Analysis

Quantitative analysis of bacterial colonization was performed on FISH images following a stepwise procedure using ImageJ software complete with the MBF (McMaster Biophotonics

Facility) plugin collection [44] specifically designed for analysis of microscopy images. For each of the three channels within the original file representing the SytoBC live-dead stain, the domain Bacteria probe, and the specific group probe, quantification was performed at depths of 2, 4 and 6 μm, that were averaged together after analysis. The SytoBC live-dead stain channel, was used to differentiate fluorescence produced by bacterial cells from that of the background fluorescence or autofluorescence. Bacterial cells and colonies were detected and outlined using the nucleus counter plug-in (a part of the MBF ImageJ plugin collection) with the following settings: particle size set to 5 200, automatic subtraction of background, and application of the watershed filter. The outlined “particles”, or in this case bacterial cells, were saved to the ROI (region of interest) manager. Each image was manually checked to confirm that cells selected by the plugin were in fact bacterial cells and to add cells missed by the plugin. The rhizoplane area covered by cells stained with the SytoBC live-dead stain was then calculated. This cell coverage calculation represented both live and dead bacterial cells. Second, channel 2, the bacteria probe channel, was analyzed using the library of stored cells identified based on the SytoBC stain and saved to the ROI manager. Each cell was individually queried for detection with the domain Bacteria probe. Cells not detected with the Bacteria probe were deleted. Remaining data from channel 2 were used to calculate the total area of metabolically active bacteria cells. Third, the same step was repeated with channel 3 images for the phylum/subphylum specific probe. This step-wise colocalization strategy decreased the chance of including autofluorescence into the analysis and ensured that specific cells (*Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*) were also stained with the Bacteria probe and SytoBC nucleic acid stain.

Three quantitative measurements were then recorded as follows. First, the relative abundances of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria* were calculated by dividing the area of cells stained by each specific probe by the total area of cells stained with Bacteria probe. Second, the percentage of metabolically active bacteria was calculated by dividing the area of cells stained by the Bacteria probe by the area of cells stained by the SytoBC nucleic acid stain. And third, the bacterial rhizoplane coverage was calculated by dividing the area of cells stained by the Bacteria probe by the area of the rhizoplane, thus this measurement represents the percent of the rhizoplane colonized by metabolically active bacteria. The above calculations at 2, 4, and 6 μm depths were averaged to get a final value for each root sample.

PCA of Plants from IKMHSS Field Site

Principal component analysis (PCA) was performed on Phase 1 and Phase 3 samples, separately, in order to identify deterministic factors influencing the species composition of bacteria colonizing the rhizoplanes under different field conditions. The PCA analyses were performed using CANOCO 4.5 (Microcomputer Power, Ithaca, NY).

Microbial Phylogenetic Analysis (16S rRNA gene) of Plant Rhizosphere Substrates from IKMHSS Field Site

Rhizosphere microbial phylogenetic analysis was used to evaluate the influence of rhizosphere populations on bacterial rhizoplane colonization patterns of plants harvested from the IKMHSS field site. Microbial community profiles were generated from 16S rRNA

gene amplicons from rhizosphere-influenced material collected from each plant harvested from Phase 1 and Phase 3, as described previously [17]. From the rhizosphere-influenced sample, a 0.5 g subsample was subjected to DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals; Santa Ana, CA) following the manufacturer's protocol with modifications to enhance DNA yield as outlined in Valentín-Vargas et al. [7]. Library preparation and sequencing were performed at Argonne National Laboratory (Chicago, IL). Library preparation followed a single-step PCR protocol presented by Caporaso et al. [45] using primers 515f and 806r to amplify the V4 region of the 16S rRNA gene. Sequencing was carried out in a single lane on an Illumina Mi-Seq using v2 chemistry and bidirectional amplicon sequencing (2 x 151bp).

Raw sequence reads were processed using the open source software package QIIME v1.9 (Quantitative Insights into Microbial Ecology; www.qiime.org). Forward and reverse reads were joined, using a minimum overlap of 30 bp followed by quality filtering using default QIIME parameters and demulitplexing. Default QIIME quality filtering parameters include: removal of the primer/barcode from the sequence, and removal of all sequences 1) with a Phred quality score <4; 2) containing any ambiguous (N) base calls; and 3) having a length of < 0.75 of original length post quality filtering. After quality filtering, a total of 1,863,169 high quality sequences, with an average number of 116,448 ± 30,667 sequence reads per sample remained. Using a sequence cutoff of 30,000 sequences, one sample (Row4b) from Phase 3 was removed from further analysis. Sequences were clustered into operational taxonomic units (OTUs) of greater than or equal to 97% similarity using UCLUST [46]. During DNA extraction, blanks that contained no tailings sample were extracted alongside each batch of samples for quality control and subsequently sequenced. OTUs contained in the sequenced blanks were removed from the associated samples extracted in the same batch to minimize influence of contamination. Representative sequences from each OTU were aligned using PyNAST [47] and assigned taxonomy using the UCLUST classifier and Greengenes 16S rRNA gene database [48]. Sequence data have been deposited in the NCBI Sequence Read Archive (SRA) database under the accession number SRR4462508.

Results

Mesocosm Study

The first phase of this study represents a temporal analysis of buffalo grass plants grown in mesocosms in 15% compost-amended tailings under highly controlled greenhouse conditions. Pore water chemistry and plant establishment were monitored during the course of the experiment as explained previously [7,34]. Here we examine bacterial rhizoplane colonization of plants harvested at 3, 6, and 9 months during acidification of metalliferous pyritic tailings. A significant temporal decrease was observed for both the relative abundance and rhizoplane coverage of metabolically active bacteria colonizing the root surface at 6 months ($p < 0.05$) (Table 1). A concurrent increase in the relative abundance of *Gammaproteobacteria* occurred at 6 months ($p < 0.05$) while *Alphaproteobacteria* and *Actinobacteria* relative abundance did not change significantly over the 9 months evaluated (Table 1). These observed temporal changes in bacterial rhizoplane colonization were associated with significant decreases in percent plant cover at 6 months ($p < 0.05$) and pH at

9 months ($p < 0.05$) (Table 1). Pore water DOC, DN, and EC showed no significant change with time (Table 1). These results reveal that temporal changes in relative abundance and rhizoplane coverage of metabolically active bacteria, and the relative abundance of *Gammaproteobacteria* on the rhizoplane were associated with changes in substrate pH and plant growth status.

Rhizoplane Colonization of Buffalo Grass from the IKHMSS Field Trial

After using FISH to observe buffalo grass rhizoplane bacterial colonization patterns during acidification of 15% compost-amended (w/w) IKHMSS mine tailings in the controlled mesocosm study, bacterial colonization of buffalo grass roots harvested in 2013 from Phase 1 and Phase 3 of the IKHMSS field trial (15% compost amended [w/w]) treatments were characterized. The IKMHSS field study is an ongoing 7-year phytostabilization field trial in Dewey-Humboldt, AZ that examines diverse strategies to facilitate plant establishment on highly, acidic pyritic mine tailings as described previously [31]. At the time of sampling, the Phase 1 and Phase 3 field trials represented three years and one year of growth, respectively. Eight plants from each trial were sampled as described in the Methods, but one Phase 3 root degraded during transport and could not be analyzed (Row 2c). Chlorophyll levels recorded from each plant at the time of sampling (atLeaf Chlorophyll meter) were used to separate the plants into two categories; high chlorophyll with values of 32 50, and low chlorophyll with values of 8 16. Chlorophyll level was used as a proxy for plant status at the time of sampling with high chlorophyll levels representing robust plants and low chlorophyll levels representing stressed plants (Table 2). Plant chlorophyll content has been shown to correspond to plant health within the same species [40]. The relative abundance of metabolically active bacteria on the rhizoplane was significantly higher for the plants with high chlorophyll levels compared to that of the plants with low chlorophyll levels ($p < 0.05$) (Table 2). This pattern is similar to the trend observed for plants from the mesocosm experiment in which plants harvested from mesocosms with significantly higher plant cover had significantly higher relative abundance of rhizoplane metabolically active bacteria (Table 1) than those plants from mesocosms of lower plant cover. The average rhizoplane coverage for metabolically active bacteria was also higher on the roots from the plants with high chlorophyll levels, however this trend was not significant. An examination of the specific phyla colonizing the rhizoplanes showed no significant trend associated with chlorophyll level.

A comparison was then made between the plants harvested from Phase 1 and Phase 3 field trials. Recall that major differences between phases include: 1) difference in time of implementation, and therefore age of plants and compost amendment, with Phase 1 starting in May 2010 and Phase 3 starting in June 2012; 2) addition of lime in Phase 3 versus no lime in Phase 1; and 3) Phase 1 seeded with mixed plant species while Phase 3 seeded with a monoculture of buffalo grass. Plants sampled from each phase included a broad range of chlorophyll content (Table 3) indicating that a comparable range of plant health was sampled from both field trials. Further results show that the relative abundance of *Actinobacteria* was significantly higher on the roots of Phase 1 plants compared to Phase 3 ($p < 0 .05$) (Table 3). The other bacterial rhizoplane colonization measurements were not significantly different for plants harvested from the two separate field trials. There was high variability in both the

percent rhizoplane coverage as well as the relative abundance of specific phyla/subphyla colonizing rhizoplanes within Phase 1 and Phase 3 (CV = 0.5 – 0.8) (Table 3). The percent of metabolically active bacteria varied little between Phase 1 and 3 (CV = 0.1 for Phase 1 and Phase 3) (Table 3). Nutrient status (TOC and TN) and pH were significantly higher ($p < 0.05$), and more favorable, in Phase 3 than Phase 1 (Table 3). Higher TOC, TN, and pH values in Phase 3 can be attributed to the more recent addition of compost. The higher pH in Phase 3 can also be due to the addition of lime in this treatment. EC was also significantly higher in Phase 3 ($p < 0.05$) (Table 3), representing the only less desirable geochemical parameter in Phase 3. Plant cover was lower and more variable (coefficient of variation [CV] = 0.6) in Phase 1 than Phase 3 (CV = 0.4), but the difference was not significant (Table 3). This trend may reflect the observed difference in geochemical parameters between Phase 1 and Phase 3, or could also be an effect of the presence of a monoculture in Phase 3 vs. polyculture in Phase 1.

Next, the impacts of rhizosphere geochemistry and plant condition were evaluated as explanatory variables for the high within treatment variability in rhizoplane colonization patterns for different plants from Phases 1 and 3. PCA was performed using the relative abundances of the different phyla/subphyla on the rhizoplanes. In Phase 1, *Alphaproteobacteria* relative abundance was negatively associated with TN, pH, and plant cover along PC1 (x axis; explaining 72.9% of the variation) (Figure 2a). *Gammaproteobacteria* relative abundance was positively associated with EC and negatively associated with plant cover. *Actinobacteria* were not highly associated with any of the plant status or geochemical parameters. Overall for Phase 1, the geochemical and plant status variables explained 97.7% of the total variation in bacterial composition of rhizoplane colonizers (Figure 2a) with plant cover followed by pH being the most significant explanatory variables. In contrast, in Phase 3, *Alphaproteobacteria* relative abundance on rhizoplanes was positively associated with TN, TOC, pH, chlorophyll, and plant cover along PC1 (83.3% of the variation). *Gammaproteobacteria* and *Actinobacteria* were positively associated with EC and negatively associated with pH, however the short *Actinobacteria* vector indicate less significance of this phylum relative to the other subphyla. In Phase 3, the geochemical and plant status parameters explained 95.8% of the total variation in bacterial composition (Figure 2b), with pH, TOC, and EC being the largest drivers. In summary, *Alphaproteobacteria* relative abundance on rhizoplanes was positively associated with pH, TN, and plant cover in Phase 3, but negatively associated with the same parameters in Phase 1. Second, strong negative associations are observed for *Gammaproteobacteria* relative abundance with plant cover in Phase 1 and with pH in Phase 3 (Figures 2a and 2b). These results suggest that *Gammaproteobacteria* rhizoplane colonization increases under conditions of stress, a finding that is consistent with results from the mesocosm study which showed a significant increase in relative abundance of *Gammaproteobacteria* concurrent with a decrease in buffalo grass plant cover from 73% to 10% (Table 1).

Associations between the Rhizosphere Microbial Community and Rhizoplane Colonization Patterns

Phylogenetic profiles of the rhizosphere bacterial communities were explored as potential drivers of the observed rhizoplane colonization patterns. The rhizosphere communities

represent the source from which the rhizoplane-colonizing bacteria might migrate or be recruited by the plant to the rhizoplane. As observed previously, the relative abundance of *Alphaproteobacteria* colonizing the rhizoplane varied with pH, however pH had no impact on the relative abundance of *Alphaproteobacteria* in the rhizosphere in Phase 1 or Phase 3 (Figure 3). In contrast, the *Alphaproteobacteria* phylogenetic profiles at the family level were strikingly different between the two phases and varied significantly with pH (Figure 3). In Phase 1, the buffalo grass rhizosphere was dominated by the family *Acetobacteraceae* at low pH (Figure 3c). Overall, the Phase 3 buffalo grass rhizospheres had a significantly lower relative abundance of *Acetobacteraceae* ($p < 0.05$) than Phase 1 and, at lower pH, had the highest relative abundance of *Sphingomonadaceae*. At higher pH (> 6), there was a consistent pattern in both Phase 1 and Phase 3 of increased relative abundance *Hyphomicrobiaceae,Phyllobacteriaceae*, and *Rhizobiales* as well as an unknown *Alphaproteobacteria* in the rhizosphere (Figures 3C and 3d). Taken together, these results suggest that *Acetobacteraceae* was the dominant family influencing *Alphaproteobacteria* rhizoplane colonization under low pH conditions, whereas *Hyphomicrobiaceae, Phyllobacteriaceae, Rhizobiales,* and the unknown *Alphaproteobacteria* were the primary groups potentially influencing rhizoplane colonization at pH > 6.

Gammaproteobacteria relative abundance on rhizoplanes was influenced most by plant cover in Phase 1 and pH in Phase 3 (Figures 2a and 2b). The greatest relative abundance was observed in areas of low plant cover (Figure 4a) during Phase 1, and low pH in Phase 3 (Figure 5a). Similar to *Alphaproteobacteria*, the high variability of *Gammaproteobacteria* on the rhizoplane is not paralleled by a similar variability in the relative abundance of *Gammaproteobacteria* in the rhizosphere microbial community (Figures 4b and 5b). However, major differences were observed in the phylogenetic composition of the rhizosphere *Gammaproteobacteria* communities of Phase 1 and Phase 3 plants. *Xanthomondaceae* and *Sinobacteraceae* comprised between 71.7 99.8% of total *Gammaproteobacteria* for all Phase 1 plant rhizospheres with the exception of the plant harvested from Plot 10a (30.4% relative abundance) (Figure 4b). In Phase 3, the *Gammaproteobacteria* were more diverse and also had large representation from the *Alteromonadaceae* and *Pseudomonadaceae* families and the *Chromatiales* order (Figure 5b). Of interest is the unique *Gammaproteobacteria* rhizosphere community profile for the plant harvested from Row 2b that is associated with the lowest rhizosphere pH, the highest relative abundance of rhizoplane colonization, and the highest relative abundance of *Acidithiobacillaceae* in the rhizosphere. The observed variation in rhizosphere *Gammaproteobacteria* community phylogenetic profiles for Phase 1 and Phase 3 confirms that distinct groups of *Gammaproteobacteria* influence colonization of the rhizoplanes of buffalo grass plants under the different conditions in these two field trials.

Discussion

This study provides the first evidence for a deterministic pattern of bacterial rhizoplane colonization of plants grown in metalliferous mine tailings. The results show that the relative abundance and rhizoplane coverage of metabolically active bacteria and the relative abundance of specific subphyla reflect substrate geochemistry and rhizosphere community composition. As will be discussed below, these findings suggest potential roles that the

bacteria colonizing the rhizoplane play in plant adaptation and survival under the extreme conditions of the mine tailings. Knowledge of these relationships can be exploited to develop novel technologies to advance the long-term success of mine-tailing phytostabilization.

Rhizoplane-associated metabolically active bacteria

It is a significant finding that the relative abundance of metabolically active bacteria on the rhizoplane correlates with plant health. In the mesocosm study, as plant cover significantly decreased between 3 and 6 months, there was a coinciding decrease in relative abundance of metabolically active bacteria colonizing the rhizoplane (Table 1). We hypothesize that the degree of plant cover is an indicator of plant health and establishment, suggesting a possible link between plant health and abundance of metabolically active bacteria. To further test this hypothesis, plants harvested from an on-going mine-tailings phytostabilization field study were categorized according to chlorophyll levels as a measure of plant status. More vigorous plants, with high leaf chlorophyll levels, were found to have a significantly higher relative abundance of metabolically active bacteria ($p < 0.05$), supporting our hypothesis that the relative abundance of metabolically active bacteria on the rhizoplane correlates with plant health. This correlation is likely driven by a combination of increased plant root exudation of healthy plants creating a more abundant food source for bacteria, and in exchange, more active bacteria providing beneficial services that improve plant fitness. In fact, previous studies have shown that plant health and growth can lead to an increase in root exudation which has been found to positively correlate with rhizosphere microbial activity [49,50,51].

Subphyla

The significant relationship observed between plant health and rhizoplane-colonizing bacteria led to a more in-depth interrogation into relationships between the relative abundance of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria* colonizing the rhizoplane and geochemical and plant status parameters. Most pronounced is the contrasting pattern of *Alphaproteobacteria* relative abundance, which is negatively associated with pH and TN in Phase 1, and is positively associated with pH, TOC, and TN in Phase 3. This interesting contrast in pattern reflects the significantly different geochemical conditions of Phase 1 and Phase 3 ($p < 0.05$) (Table 3), most notable between pH, TOC, and TN. As mentioned previously, Phase 1 was implemented in 2010 and Phase 3 in 2012. At the time of sample collection in 2013, the compost amendment in Phase 3 was still abundantly present helping to maintain a buffering effect and source of organic matter. However, Phase 1 compost levels were lower, leading to lower organic matter content and a decrease in buffering capacity that was associated with re-acidification of the tailings material (Table 3). An increase in *Gammaproteobacteria* relative abundance was observed on the rhizoplane under stressed conditions, as evidenced by the negative association with plant cover in Phase 1 and pH in Phase 3, which will be discussed below. Finally, *Actinobacteria* colonization on the root surface was greater in Phase 1 than Phase 3 (Table 3), but no major associations between colonization patterns and geochemical parameters were observed for this phylum. Because no major patterns between *Actinobacteria* and geochemical conditions were observed in Phase 1 and Phase 3, this phylum was not focused on for further analysis.

Variations in *Alphaproteobacteria* and *Gammaproteobacteria* phylogenetic composition within the rhizosphere substrate material reveals striking patterns that correspond with rhizoplane colonization patterns. The relative abundance of both subphyla colonizing the rhizoplane shows high variation; however, this variation is not explained by differences in the respective abundances of these groups in the Phase 1 and Phase 3 rhizosphere material. Despite the lack of variation in relative abundance, the phylogenetic profiles of the rhizosphere *Alphaproteobacteria* and *Gammaproteobacteria* in Phase 1 and 3 are quite distinct and vary with the specific geochemical conditions shown to influence the root colonization patterns of these two taxa. These results suggest that differential rhizoplane colonization patterns could be explained by either selective recruitment from the distinct rhizosphere communities by the plant or preferential colonization of the plant rhizoplane by rhizosphere bacteria.

Alphaproteobacteria—The relative abundance of *Alphaproteobacteria* rhizoplane-colonization is negatively associated with pH in Phase 1 and positively associated with pH in Phase 3. Phylogenetically distinct rhizosphere *Alphaproteobacteria* communities were also observed in Phase 1 vs. Phase 3. *Alphaproteobacteria* communities in the Phase 1 rhizosphere are increasingly dominated by acidophilic *Acetobacteraceae* as the pH declines, of which *Acidiphilium* is the dominant genus (39.5 91.7%). Species within the genus *Acidiphilium* are known to reduce Fe, and at least one species, *Acidiphilium mulitvorum*, is known to oxidize As(III) [52,53]. In a previous analysis of buffalo grass plants grown in the IKMHSS tailings, up to 27.4% of rhizoplane *Alphaproteobacteria* were co-localized with Fe/As plaques on buffalo grass roots from Plot 24 in Phase 1 [29]. Taken together, these data give rise to the speculation that *Acetobacteraceae*, including *Acidiphilium*, are colonizing the rhizoplane and participating in Fe and/or As cycling. In contrast, the Phase 3 *Alphaproteobacteria* phylogenetic communities are dominated by *Hyphomicrobiaceae*, *Phyllobacteriaceae*, and other *Rhizobiales* at higher, more neutral, pH, when the relative abundance of *Alphaproteobacteria* on the rhizoplane is high. According to 16S rRNA gene sequencing results, the family *Hyphomicrobiaceae* primarily consists of the genus *Devosia* (43.0 92.6%), a known PGPB with the capacity for nitrogen-fixation [54]. The family *Phyllobacteriaceae* also contains many PGPB species capable of nitrogen-fixation [52]. It is possible that these PGPB are the *Alphaproteobacteria* colonizing the rhizoplanes in Phase 3, which would explain the positive association of *Alphaproteobacteria* on the rhizoplane with the favorable nutrient status, as indicated by TN and TOC, and more neutral conditions. The stark difference in *Alphaproteobacteria* phylogenetic profiles in Phase 1 and Phase 3 coupled with the opposing trend of rhizoplane-colonizing *Alphaproteobacteria* relative abundance in relation to pH suggest that plant-microbe associations fluctuate with changing geochemical conditions.

Gammaproteobacteria—Phylogenetic profiles within *Gammaproteobacteria* rhizosphere communities in Phase 1 and Phase 3 also reveal an interesting trend in relation to *Gammaproteobacteria* rhizoplane colonization. In the more stressed conditions of low pH and low plant cover where there is a higher relative abundance of *Gammaproteobacteria* colonizing the rhizoplane, *Acidithiobacillaceae* (100% belonging to the genus *Acidithiobacillus*) are present in the rhizosphere at a higher relative abundance, indicating

that these may be selectively recruited by the plant or preferentially colonizing the rhizoplane under these conditions. Species within the genus *Acidithiobacillus*, most notably *A. ferrrooxidans* and *A. thiooxidans*, are known to oxidize Fe and S, respectively [52]. Using the method for quantifying metal(loid) and bacterial colocalization on rhizoplanes presented in Honeker et al. [17], we found that 12.4 – 47.9% of *Gammaproteobacteria* colocalized with Fe on rhizoplanes of buffalo grass from the mesocosm study at 6 months, at the time that plant cover had sharply decreased (Supp. Figure 1). This high degree of association between *Gammaproteobacteria* and Fe suggest that *Acidithiobacillus* may comprise a portion of the *Gammaproteobacteria* colonizing the rhizoplane and contributing to ferric Fe plaque formation. It is interesting to point out that root sample Plot 24b, which was associated with an acidic pH and low plant cover, had high abundance of *Alphaproteobacteria* and *Gammaproteobacteria* (37.8 and 50.3%, respectively). As previously mentioned, both of these subphyla also exhibited a high degree of association with Fe on the buffalo grass rhizoplane. Under acidic conditions, bacterially-mediated transformation of Fe is favored, while abiotic Fe oxidation kinetically occurs relatively slowly. These conditions promote the creation of poorly crystalline Fe(III) oxides, such as ferrihydrite, which were previously shown to be the mineral phase constituting the Fe plaque on rhizoplanes from IKMHSS [17]. Therefore, a possible scenario is that bacterial-mediated rapid Fe-cycling in the root zone and on the rhizoplane may be occurring on the buffalo grass roots, involving the alphaproteobacterium *Acidiphilium* as the Fe reducer and the gammaproteobacterium *Acidithiobacillus* as the Fe oxidizer [55]. Probes can be designed to test this hypothesis in future work.

Conclusions

The results of this study reveal that bacterial rhizoplane colonization patterns are variable within a single plant species in reflection of geochemical and plant status parameters. The combined use of iTag 16S rRNA bacterial gene community analysis of rhizosphere substrate populations and FISH profiling of rhizoplane bacteria enabled a characterization of the bacterial taxa colonizing the rhizoplane, with possible explanations for influences that these plant-microbe associations may have on plant health under harsh environmental conditions. Taken together, the results of this study suggest a model in which buffalo grass roots from neutral compost-amended tailings material are colonized predominantly by PGPB, whereas roots from plants surviving in a more highly acidic, weathered tailings/compost mixture with significantly lower TOC and TN levels have colonization patterns dominated by metal-cycling bacteria. To confirm this model, future work is needed using family, genus, and species specific FISH probes to target the potential PGPB (*Phyllobacteriaceae, Hyphomicrobiaceae*) and metal-cyclers (*Acidiphilium, Acidithiobacillus)*. The significance of these patterns is not known, but we hypothesize that they influence plant survival. The weathering of the compost/tailings mixture occurs over time following the erosion and mineralization of compost amendments which lead to re-acidification and reduced nutrient levels. The characterization of specific plant-microbe associations during phytostabilization provides clues for understanding plant survival in the extreme environment of metalliferous mine tailings that can be exploited to develop bacterial inoculum-based technologies to help boost plant health under various suboptimal field conditions. Furthermore, these associations

may provide insights for evaluating the success and establishment of plants during phytostabilization of metalliferous mine tailings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Scott White for his work in designing and implementing the IKHMSS field study. We thank Steven Schuchardt, president of North American Industries, for providing access to the IKMHSS site and help with irrigation. We thank all volunteer students from Environmental Microbiology, Environmental Biochemistry, and Contaminant Transport Labs for their help during field implementation and sampling trips, and especially Corin Hammond for her dedicated work in helping to organize as well as participate in these sampling trips. We also thank Alexis Valentín-Vargas for collecting and fixing buffalo grass root samples from the mesocosm study that were used in the current study. This work was supported by the National Institute of Environmental and Health Sciences (NIEHS) Superfund Research Program (SRP) grant P42 ES004940 and R01 ES01709 and the National Science Foundation Graduate Research Fellowhip Program (NSF GRFP) grant DGE-1143953.

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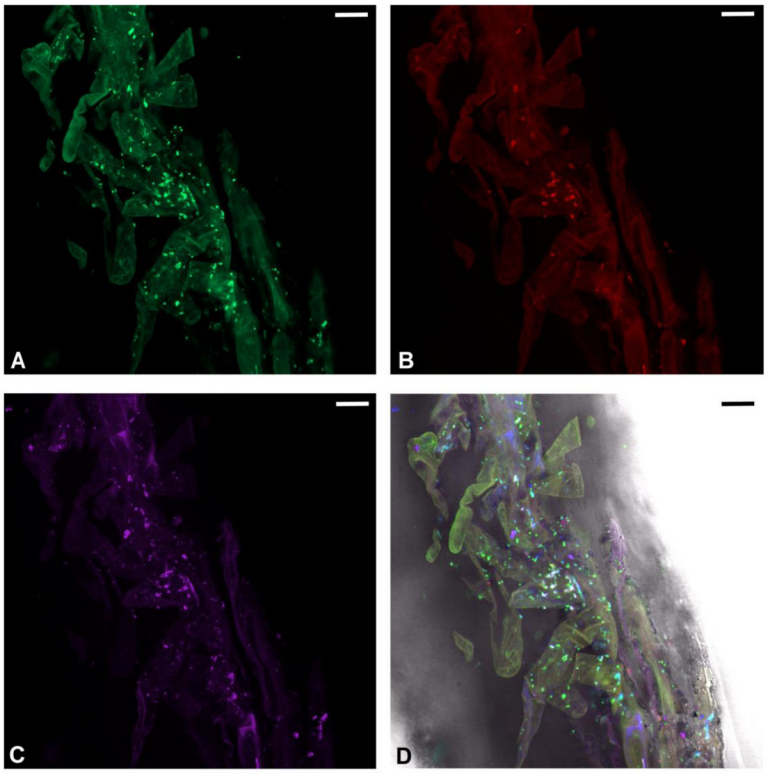

Fig. 1.

Buffalo grass root processed using A) nucleic acid stain (SytoBC) to target all Bacteria, B) fluorescence in situ hybridization (FISH) probe to target metabolically active Bacteria (Eub338), and C) FISH probe to target *Alphaproteobacteria* (Alf968). D) Overlay of fluorescence and light images, where *Alphaproteobacteria* cells appear white due to colocalization between the SytoBC nucleic acid stain and the Eub338 and Alf968 FISH probes. Roots were viewed and imaged with a Zeiss 510 Meta confocal laser scanning microscope at 63x magnification. Scale bars represent 10μm.

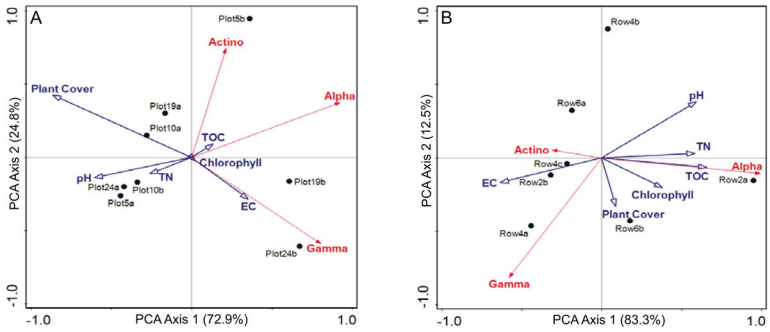

Fig. 2.

Principle Component Analysis (PCA) showing relationships between bacterial communities colonizing buffalo grass roots from A) Phase 1 and B) Phase 3 of the IKMHSS field trial. Red vectors depict the *Actinobacteria* (Actino), *Alphaproteobacteria* (Alpha), and *Gammaproteobacteria* (Gamma) colonizing the rhizoplanes and the blue arrows depict the relationship between the geochemical (pH, electrical conductivity [EC], total organic carbon [TOC], and total nitrogen [TN]) and plant status (plant cover and chlorophyll) parameters and bacterial community composition. Overall, the direction and magnitude of the red and blue vectors represent the presence of and strength of associations between specific phyla/ subphyla, geochemical, and plant status parameters. The total variance explained by each axis is shown in parentheses.

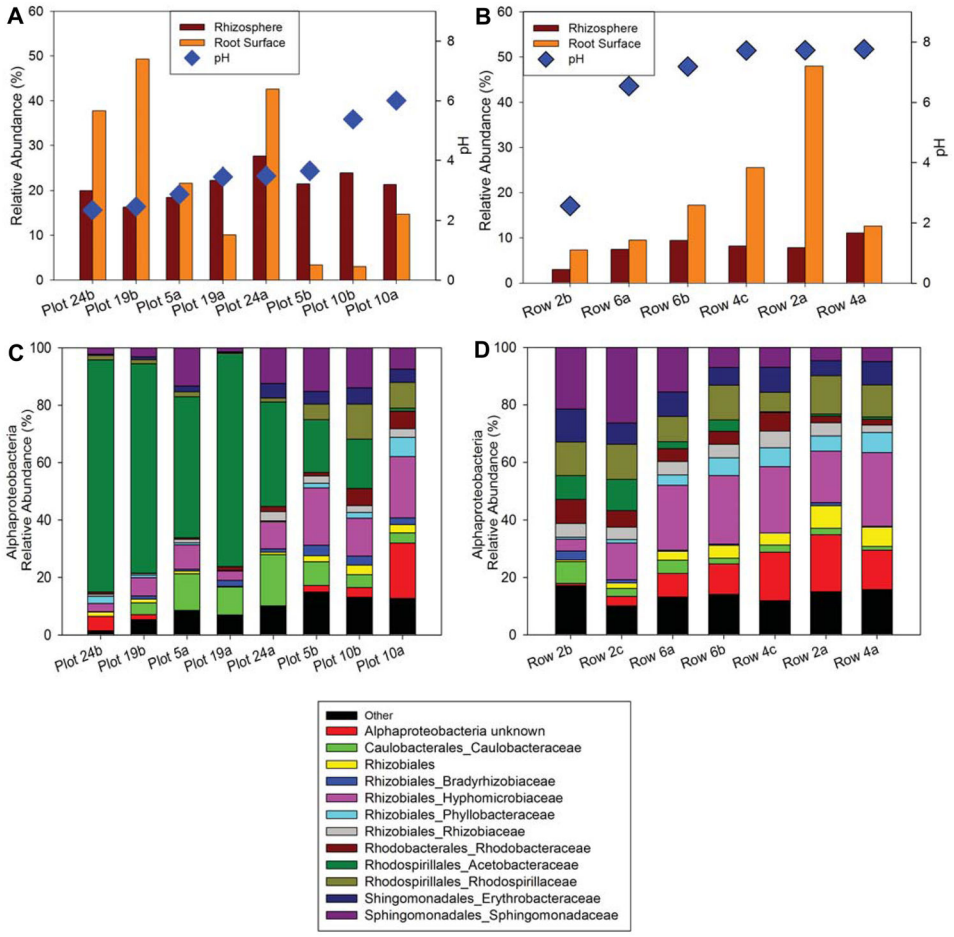

Fig. 3.

Relative abundance of *Alphaproteobacteria* on buffalo grass rhizoplane and in the rhizosphere from the of the IKMHSS field trial, as assessed using fluorescence in situ hybridization and 16S rRNA gene amplicon sequencing, respectively for A) Phase 1 and B) Phase 3. *Alphaproteobacteria* phylogenetic profiles of buffalo grass rhizosphere for C) Phase 1 and D) Phase 3. Samples arranged from left to right in order of increasing pH within each phase. pH values are representative of the surface substrate collected directly adjacent to each plant.

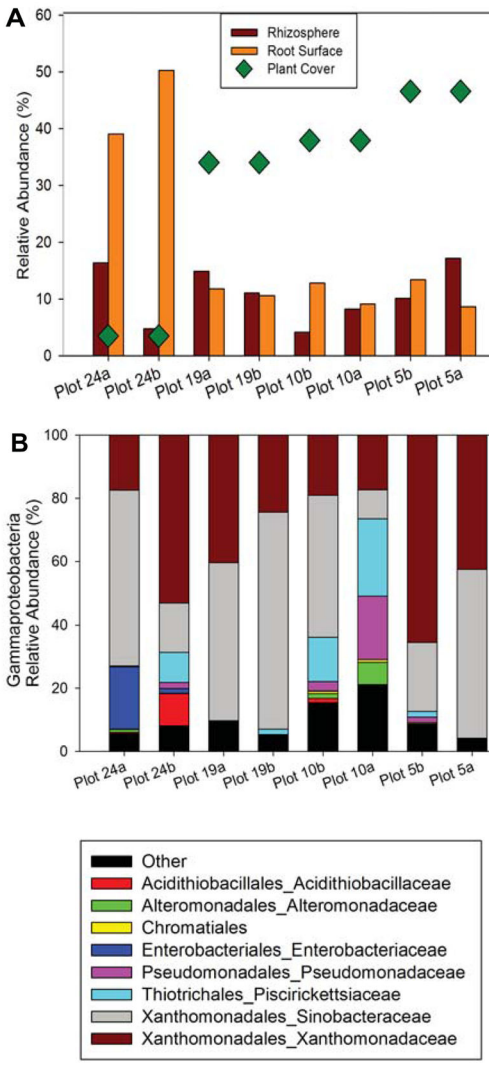

Fig. 4.

A) IKMHSS field trial Phase 1 relative abundance of *Gammaproteobacteria* on buffalo grass rhizoplanes and in the rhizosphere, as assessed using fluorescence in situ hybridization and 16S rRNA gene amplicon sequencing, respectively. B) Phase 1 *Gammaproteobacteria* phylogenetic profile of buffalo grass rhizosphere. Samples are arranged from left to right in order of increasing plant cover of the plot from which plant was collected.

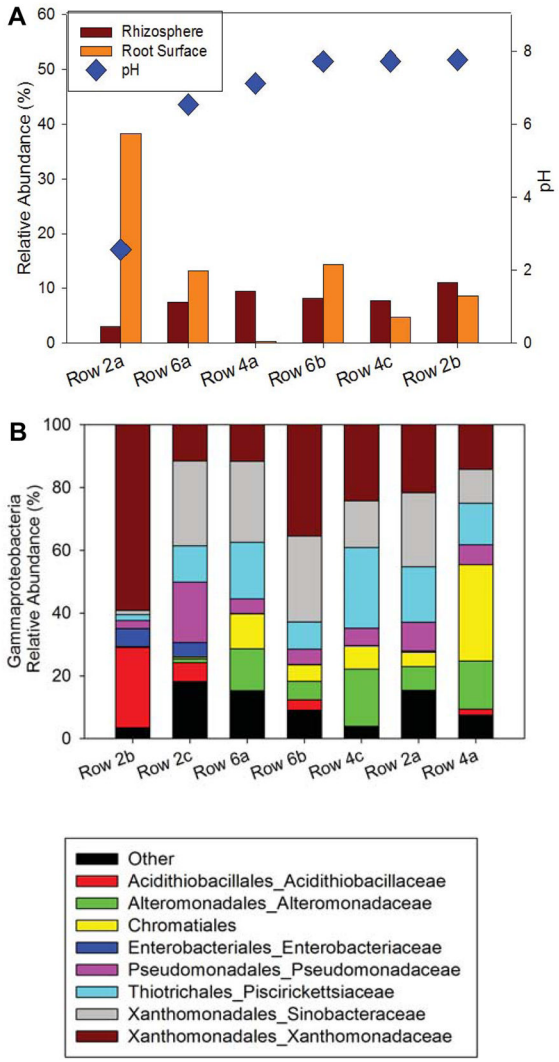

Fig. 5.

A) IKMHSS field trial Phase 3 relative abundance of *Gammaproteobacteria* on buffalo grass rhizoplanes and in the rhizosphere, as assessed using fluorescence in situ hybridization and 16S rRNA gene amplicon sequencing, respectively. B) Phase 3 *Gammaproteobacteria* phylogenetic profile of buffalo grass rhizosphere. Samples arranged from left to right in order of increasing pH of surface substrate collected directly adjacent to each plant.

Table 1

Mesocosm study geochemical, plant status, and rhizoplane bacterial colonization data. Values represent averages of buffalo grass mesocosm replicates with standard deviations (n=3 at each time point) and coefficients of variation in parentheses.

Parameters		3 months	6 months	9 months
Geochemical[c]	pH	**5.7** ± 0.5 (0.1)[a]	**6.3** ± 0.5 (0.1)[a]	**4.1** ± 0.2 (0.0)[b]
	DOC (mg L^{-1})	**1934.0** ± 1635.0 (0.8)	**844.0** ± 305.0 (0.2)	**252.0** ± 57.0 (0.5)
	DN (mg L^{-1})	**172.6** ± 175.1 (1.0)	**222.3** ± 104.1 (0.5)	**104.2** ± 68.9 (0.7)
	EC (dS m^{-1})	**30.4** ± 23.8 (0.8)	**34.3** ± 6.5 (0.2)	**30.5** ± 4.3 (0.1)
Plant Status	Plant Cover (%)	**72.8** ± 16.2 (0.2)[a]	**9.7** ± 7.8 (0.8)[b]	**17.7** ± 23.4 (1.3)[b]
Rhizoplane bacterial colonization	MAB[d] (%)	**45.8** ± 4.0 (0.09)[a]	**25.2** ± 8.1 (0.3)[b]	**19.6** ± 7.6 (0.4)[b]
	RC[e] (%)	**0.4** ± .1 (0.3)[a]	**0.1** ± 0.1 (0.7)[b]	**0.1** ± 0.1 (0.7)[b]
	Alapha.[f] (%)	**21.4** ± 21.2 (0.8)	**13.7** ± 1.3 (0.2)	**20.9** ± 10.1 (0.4)
	Gamma.[g] (%)	**8.4** ± 3.3 (0.4)[a]	**19.8** ± 2.6 (0.1)[b]	**16.9** ± 1.7 (0.1)[b]
	Actino.[h] (%)	**30.9** ± 20.8 (0.8)	**46.4** ± 7.3 (0.2)	**43.7** ± 15.5 (0.4)

DOC = dissolved organic carbon, DN = dissolved nitrogen, EC = electrical conductivity

[a,b] Different letters represent significant differences between single variables at different time points (ANOVA, $p < 0.05$; Tukey-Kramer HSD test).

[c] Geochemical parameters characterize pore water samples collected at depths of 5 and 15 cm

[d] MAB (metabolically active bacteria) % = area of Bacteria (probe Eub338 mix) / area of total live and dead bacteria (StytoBC nucleic acid stain) * 100

[e] RC (root coverage) % = area of Bacteria (probe Eub338 mix) / area of root surface *100

[f] Alpha. (*Alphaproteobacteria*) % = area of Alpha. (probe Alf968) / area of Bacteria (probe Eub338 mix) * 100

[g] Gamma. (*Gammaproteobacteria*) % = area of Gamma. (probe Gam42a) / area of Bacteria (probe Eub338 mix) * 100

[h] Actino. (*Actinobacteria*) % = area of Actino. (probe HGC69a) / area of Bacteria *100

Table 2

IKMHSS field study rhizoplane bacterial colonization data for plants with high (32–50) and low (8–16) chlorophyll levels [a]. Values represent averages with standard deviation and coefficient of variation in parentheses.

Rhizoplane bacterial colonization	High chlorophyll n=8	Low chlorophyll n=7
MAB[b] (%)	**66.3** ± 4.3 (0.1) *	**59.3** ± 6.9 (0.1) *
RC[c] (%)	**1.2** ± 0.7 (0.6)	**0.7** ± 0.3 (0.5)
Alapha.[d] (%)	**23.1** ± 14.8 (0.6)	**18.7** ± 18.0 (1.0)
Gamma.[e] (%)	**13.7** ± 10.7 (0.8)	**20.1** ± 17.5 (0.9)
Actino.[f] (%)	**6.8** ± 5.2 (0.8)	**10.8** ± 6.3 (0.6)

* Significant difference between high and low chlorophyll plants (t-test, $p < 0.05$)

[a] AtLEAF Chlorophyll Meter (measures light transmittance)

[b] MAB (metabolically active bacteria) % = area of Bacteria (probe Eub338 mix) / area of total live and dead bacteria (StytoBC nucleic acid stain) * 100

[c] RC (root coverage) % = area of Bacteria (probe Eub338 mix) / area of root surface * 100

[d] Alpha. (*Alphaproteobacteria*) % = area of Alpha. (probe Alf968) / area of Bacteria (probe Eub338 mix) * 100

[e] Gamma. (*Gammaproteobacteria*) % = area of Gamma. (probe Gam42a) / area of Bacteria (probe Eub338 mix) * 100

[f] Actino. (*Actinobacteria*) % = area of Actino. (probe HGC69a) / area of Bacteria (probe Eub338 mix) * 100

Table 3

IKMHSS field study geochemical, plant status, and rhizoplane bacterial colonization data for Phase 1 (initiated in 2010) and Phase 3 (initiated in 2012). Data was collected in 2013 and values represent averages with standard deviation and coefficient of variation in parentheses.

Parameters		Phase 1 n=8	Phase 3 n=7
Geochemical[a]	pH	**3.7** ± 1.3 (0.4) *	**6.3** ± 2.0 (0.3) *
	TOC (g/kg)	**49.8** ± 11.8 (0.2) *	**97.9** ± 44.0 (0.5) *
	TN (g/kg)	**3.8** ± 0.7 (0.2) *	**7.7** ± 2.0 (0.3) *
	EC (dS m^{-1})	**5.0** ± 2.7 (0.5) *	**8.9** ± 3.3 (0.4) *
Plant status	Plant Cover (%)	**30.5** ± 17.4 (0.6)	**48.2** ± 17.2 (0.4)
	Leaf Chlorophyll	**25.7** ± 15.0 (0.6)	**26.3** ± 16.6 (0.6)
Rhizoplane bacterial colonization	MAB[b] (%)	**62.1** ± 5.3 (0.1)	**64.1** ± 8.0 (0.1)
	RC[c] (%)	**0.8** ± 0.4 (0.6)	**1.2** ± 0.7 (0.6)
	Alpha.[d] (%)	**22.8** ± 18.2 (0.8)	**19.0** ± 14.1 (0.7)
	Gamma.[e] (%)	**19.5** ± 15.9 (0.8)	**13.5** ± 12.2 (0.6)
	Actino.[f] (%)	**12.0** ± 5.6 (0.5) *	**4.8** ± 3.5 (0.7) *

TOC = total organic carbon, TN = total nitrogen, EC = electrical conductivity

* Significant difference between Phase 1 and Phase 3 (t-test, $p < 0.05$)

[a] Geochemical parameters characterize surface substrate sample from top 20cm

[b] MAB (metabolically active bacteria) % = area of Bacteria (probe Eub338 mix) / area of total live and dead bacteria (StytoBC nucleic acid stain) * 100

[c] RC (root coverage) % = area of Bacteria (probe Eub338 mix) / area of root surface * 100

[d] Alpha. (*Alphaproteobacteria*) % = area of Alpha. (probe Alf968) / area of Bacteria (probe Eub338 mix) * 100

[e] Gamma. (*Gammaproteobacteria*) % = area of Gamma. (probe Gam42a) / area of Bacteria (probe Eub338 mix) * 100

[f] Actino. (*Actinobacteria*) % = area of Actino. (probe HGC69a) / area of Bacteria (probe Eub338 mix) * 100